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Molecular characterization of the enniatin synthetase gene encoding a multifunctional enzyme catalysing *N*-methyldepsipeptide formation in *Fusarium scirpi*

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Summary

The gene encoding the multifunctional enzyme enniatin synthetase from *Fusarium scirpi* (*esyn1*) was isolated and characterized by transcriptional mapping and expression studies in *Escherichia coli*. This is the first example of a gene encoding an *N*-methyl peptide synthetase. The nucleotide sequence revealed an open reading frame of 9393 bp encoding a protein of 3131 amino acids (M_r 346 900). Two domains designated EA and EB within the protein were identified which share similarity to each other and to microbial peptide synthetase domains. In contrast to the *N*-terminal domain EA, the carboxyl terminal domain EB is interrupted by a 434-amino-acid portion which shows local similarity to a motif apparently conserved within adenine and cytosine RNA and DNA methyltransferases and therefore seems to harbour the *N*-methyltransferase function of the multienzyme.

Introduction

Enniatins belong to the class of *N*-methylated cyclopeptides produced mainly by actinomycetes and fungi. Some compounds of this class have previously attracted attention because of their important pharmaceutical properties: thus, cyclosporins are immunosuppressive, bouvardins are cytostatic, and didemmins are both immunosuppressive and cytostatic. Enniatins differ from these peptide compounds in that they also contain ester bonds, because of the presence of D-2-hydroxyisovaleric acid (D-HIV) as well as a branched-chain amino acid (Fig. 1); they are therefore called depsipeptides. Enniatins are synthesized by filamentous fungi of the genus *Fusarium* and are postulated to play a role in wilt toxic events during infection of plants by enniatin-producing fusaria (Walton, 1990). Enniatins and structurally related depsipeptides like beauvericin and bassianolide exhibit entomopathogenic properties (Grove and Pople, 1980).

Enniatins and cyclosporins are synthesized by large multifunctional enzymes by a non-ribosomal thiotemplate mechanism (Zocher *et al.*, 1982; 1986; revieved by: Kleinkauf and von Döhren, 1990; Billich and Zocher, 1990). *N*-methyl peptide synthetases can be considered as 'hybrids' between normal peptide synthetases and *N*-methyltransferases following the same reaction mechanism with respect to substrate activation, *N*-methylation and chain elongation. The multifunctional enzyme enniatin synthetase (Esyn) consists of a single polypeptide chain of 347 kDa harbouring all catalytic functions necessary for synthesizing enniatin from D-HIV, the branched chain L-amino acid, AdoMet and ATP. Methylation of the amino acid during enniatin and cyclosporin synthesis occurs at the stage of thioesterified amino acids (Billich and Zocher, 1987; Lawen and Zocher, 1990). The covalently bound *N*-methyl amino acid residue reacts with D-HIV to form a thioesterified dipeptidol (Zocher *et al.*, 1983). The hexadepsipeptide is formed by repeated condensation of dipeptidol units and final cyclization. Enniatin synthetase carries a 4'-phosphopantetheine cofactor like *Bacillus brevis* gramicidin S synthetase B (GrsB), tyrocidine synthetase B (Gilhuus-Moe *et al.*, 1970) and the α-aminoadipate-L-cysteine-D-valine (ACV) synthetase involved in penicillin biosynthesis (Baldwin *et al.*, 1991).

Several genes encoding multifunctional peptide synthetases from filamentous fungi and bacteria involved in the biosynthesis of the ACV-tripeptide, gramicidin S and tyrocidine have been isolated and sequenced (Weckermann *et al.*, 1988; Krätzschmar *et al.*, 1989; Smith *et al.*, 1990; Diez *et al.*, 1990; MacCabe *et al.*, 1991; Gutierrez *et al.*, 1991; Turgay *et al.*, 1992). The identification of three regions within the deduced protein sequence of the ACV synthetase (ACVS) from *Penicillium chrysogenum* with high similarity to each other and to proteins which recognize and adenylate amino acids led to the assumption that each of the three ACVS domains may activate one of the constituent amino acids of the ACV tripeptide (Smith *et al.*, 1990; Diez *et al.*, 1990). This hypothesis is supported by protein sequence data obtained from the *B.*

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brevis grsB gene involved in gramicidin S biosynthesis whose product exhibits four conserved domains and catalyses the activation and condensation of four different amino acids (Turgay *et al.*, 1992).

We have isolated and sequenced the gene that encodes enniatin synthetase from *Fusarium scirpi*, which is the first example of a gene encoding a peptide synthetase capable of catalysing the formation of a methylated peptide; it shows a novel structure when compared with known multifunctional peptide synthetases.

Results

Isolation of DNA carrying the esyn1 *gene*

A cDNA library of *F. scirpi* poly $(A)^+$ RNA constructed in the bacterial expression vector λgt11 was screened with a monospecific polyclonal Esyn antiserum. Of approx. 4×10^5 plaques screened, two clones were obtained, which remained immunopositive on rescreening. To confirm that the clones represent parts of the *esyn1* gene, Northern hybridizations with total RNA of *F. scirpi* were performed using the cloned cDNA inserts as a probe. One of the clones, cES3, hybridized with a 9500-base RNA species (Fig. 2). Sequencing of clone cES3 revealed a truncated open reading frame (ORF) encoding 453 amino acids followed by a poly (A) tail. A genomic library of *F. scirpi* DNA in Lambda DASH was probed with the radiolabelled cES3 insert. This revealed clone DASH ES1. Figure 3A shows the organization of the 16 kb genomic insert of clone DASH ES1.

Transcriptional mapping and expression studies of the esyn1 *coding region*

To localize the transcription unit at the genomic clone

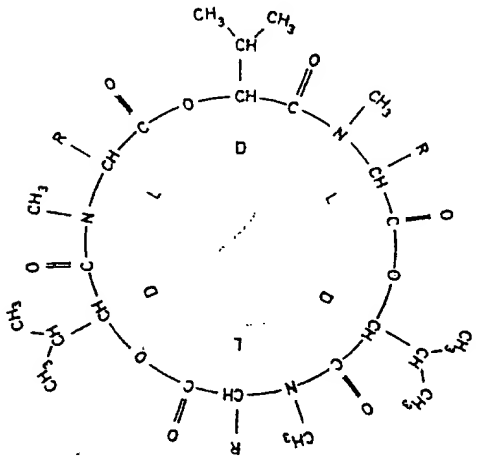

Fig. 1. Enniatin A R= $-CH(CH_3)CH_2CH_3$, Enniatin B R= $-CH_2(CH_3)_2$, Enniatin C R= $-CH_2CH(CH_3)_2$.

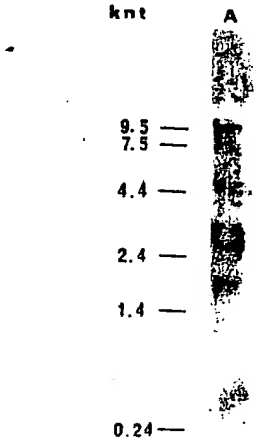

Fig. 2. Northern hybridization of total RNA from *F. scirpi* with probe cES3 (lane A). The hybridizing band corresponds to a 9500-base transcript. The positions of RNA size markers are indicated on the left.

DASH ES1 Northern hybridizations using total RNA of *F. scirpi* were carried out with several radiolabelled probes corresponding to the cDNA insert cES3 (P1) and the subcloned fragments of DASH ES1 (P2-P8) shown in Fig. 3A. The results indicated a transcript of about 9500 bases. The direction of transcription was deduced from the localization of the cES3 insert (Fig. 3A) and the direction of its truncated ORF. The 5' end of the transcript was shown to occur in the 556 bp *Bam*HI–*Bgl*I fragment located near the left DASH arm boundary.

To achieve further characterization of the large presumptive ORF on DASH ES1 and epitope mapping, different portions of the *esyn1* gene, as indicated in Fig. 3B, were subcloned in the 3' region of the *croN–lacZ* gene encoding a 116 kDa protein of pEX1,2,3 vectors (Stanley and Luzio, 1984). From a 7.1 kb *Sal*I fragment a 260 kDa protein was expressed under the control of the inducible *lac* promoter of pUC8. *E. coli* cells harbouring the expression plasmids were shown to overproduce fusion proteins with a molecular weight agreeing with the sizes of the inserted DNA fragments, when grown under inducing conditions (Fig. 4A).

Binding studies using Esyn-directed monoclonal antibodies

Two of the previously described monoclonal antibodies (mAbs) to the multifunctional enzyme Esyn, mAb 21.1 and mAb 25.91, reacted with denatured Esyn and could therefore be used in Western blot experiments. Both antibodies significantly inhibited thioester formation of the substrate amino acid L-valine (Billich *et al.*, 1987). This

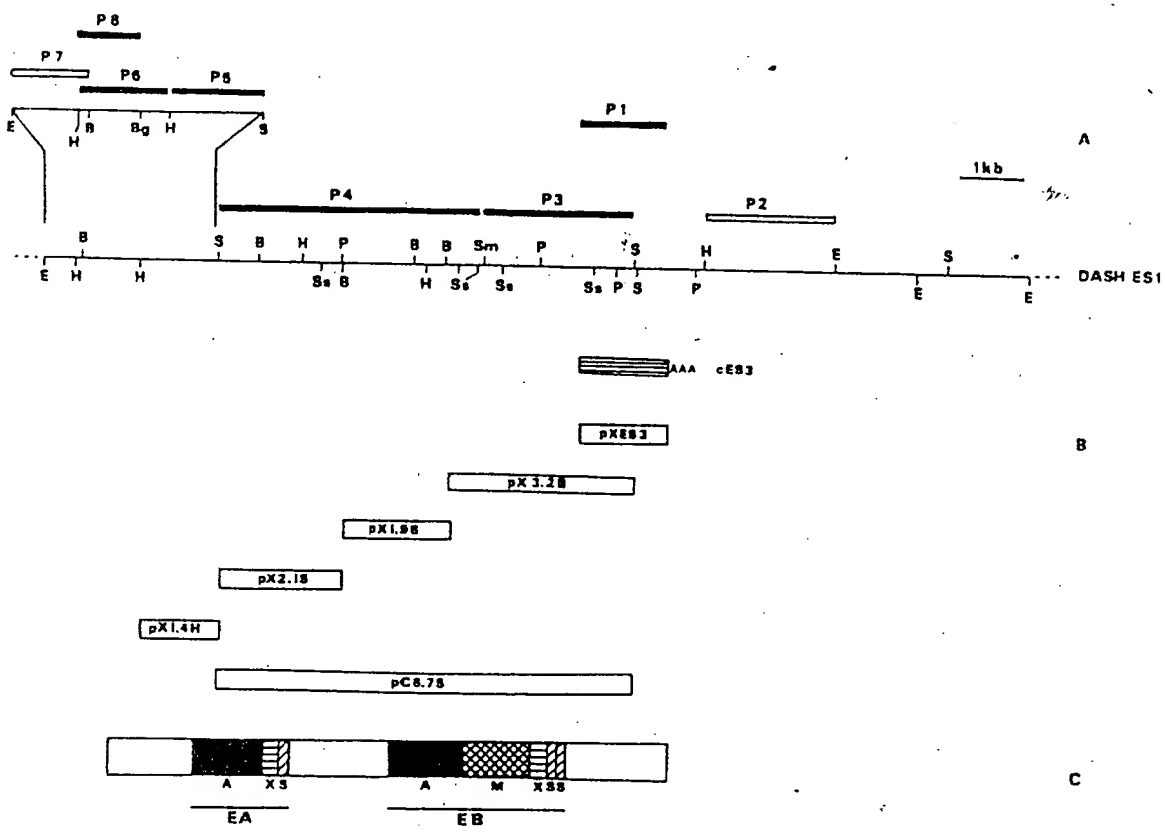

Fig. 3. Restriction map of the insert of Lambda DASH ES1 clone. The position of the cES3 clone is indicated as a striped bar. (B, *Bam*HI; Bg, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Ss, *Sst*I).
A. The DNA fragments used in Northern hybridizations to map the transcription unit of the *esyn1* gene. Black bars indicate the fragments hybridizing with the 9500-base transcript shown in Fig. 2; open bars indicate the fragments which showed no hybridization with *F. scirpi* total RNA.
B. Map of Lambda DASH ES1 subclones used for expression studies in *E. coli*. Fragments were subcloned in the 3' portion of the *lacZ* gene of pEX1-3 vectors which encode a 116 kDa cro-β-galactosidase protein (named pX) and in pUC8 (named pC6.7S).
C. Distribution of conserved regions within enniatin synthetase. Black (A), striped (X) and hatched (S, encoding L/MGGXS motif) boxes indicate regions showing high similarities to other peptide synthetases. The cross-hatched box (M) shows local similarity to methyltransferases.

prompted us to use these mAbs to investigate the *esyn1*-derived fusion proteins for the possible presence of the valine-binding site. All the fusion proteins coded by parts of the ORF on DASH ES1 were recognized by the polyclonal Esyn antiserum (Fig. 4B), whereas mAb 21.1 reacted only with proteins expressed by pC6.7S and pX1.9B (Fig. 4C). The fusion protein expressed by pX1.4H was detected only by mAb 25.91 (Fig. 4D).

Sequencing the esyn1 *coding region*

The nucleotide sequence of a 10 253 bp region encoding the entire enniatin synthetase gene has been reported to the EMBL nucleotide sequence databases and will appear under the accession number Z18755. The sequence revealed an ORF of 9393 bp starting at an ATG 330 bp downstream of the 5' *Bam*HI site, in agreement with the transcript initiation region identified by Northern hybridization and the stop codon found in the nucleotide sequence of clone cES3. This ORF has a G+C content of 53.9% which is typical for genes of filamentous fungi (Ballance, 1986). It shows compositional bias (Fickett, 1982) and the codon usage frequency is similar to that of the *Fusarium solani* f. sp. *pisi* cutinase gene (Soliday *et al.*, 1984) and the *Fusarium sporotrichioides* sesquiterpene cyclase gene (Hohn and Beremand, 1989). Although there are three further in-frame ATGs at positions –275, –236 and –209, no codon bias was found in the region outside the deduced ORF, suggesting that the predicted start codon is correct. This conclusion is corroborated by the presence of TATA-like sequences 233 (TATAAA) and 156 (TATATA) nucleotides upstream of the proposed start codon which may be important for *esyn1* gene expression (Gurr *et al.*, 1986). No evidence was found for introns in the *esyn1* gene.

The *esyn1* ORF ends at a TAG codon and several stop

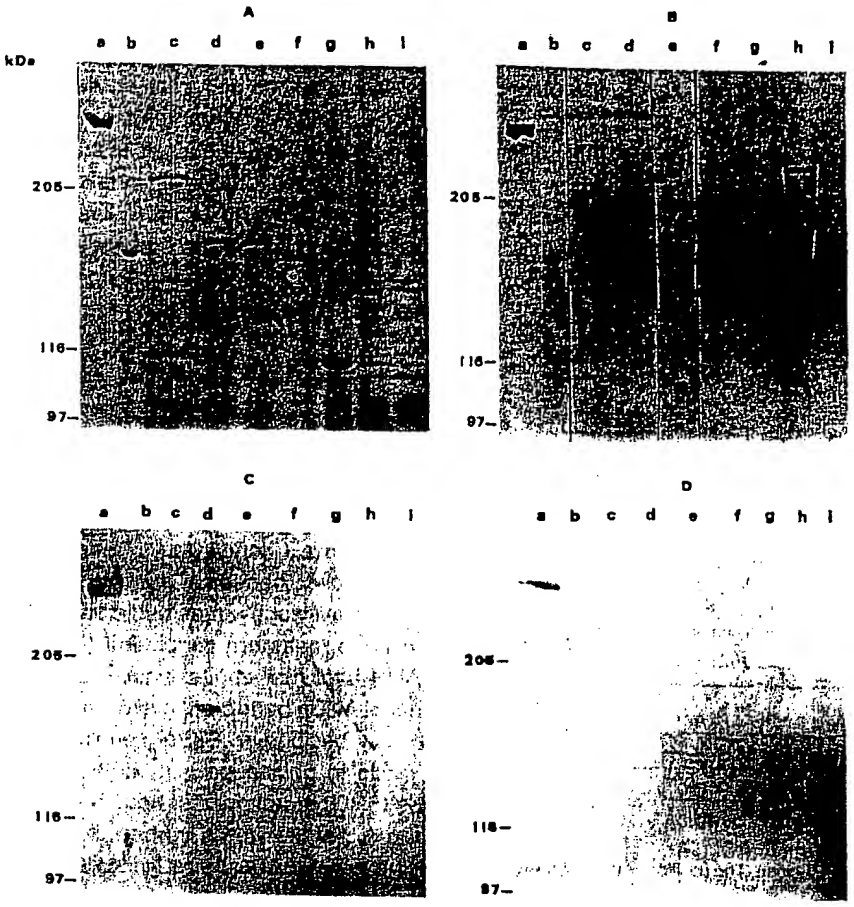

Fig. 4. Analysis of the proteins encoded by the subclones pC6.7S, pX1.4H, pX2.1S, pX1.9B, pX3.2B, and pXES3.
A. 5% polyacrylamide gel stained with Coomassie brilliant blue R. Lane a, purified Esyn; lane b, protein from pop 2136 harbouring pXES3; lanes c–g, protein extracts from pop 2136 bearing pX3.2B (lane c), pX1.9B (lane d), pX2.1S (lane e), pX1.4H (lane f), vector pEX2 (lane g); pop 2136 cells were heat-induced. Lane h, protein from JM109 harbouring pC6.7S induced with IPTG; lane i, crude extract from JM109 bearing vector pUC8.
B.–D. Immunoblots of proteins expressed by DASH ES1 and cES3 subclones. The antisera were Esyn antiserum (B), monoclonal antibody 21.1 (C), and monoclonal antibody 25.91 (D). Monoclonal antibody 21.1 recognized the 260 kDa (lane h) and 185 kDa (lane d) fusion protein expressed from pC6.7S and pX1.9B, respectively, while 25.91 recognized the 164 kDa fusion protein (lane f) expressed from pX1.4H. Purified Esyn was used as control. Molecular mass standards are indicated at the left of each panel.

codons were found in all three reading frames in the subsequent 150 bp. In the nucleotide sequence of the cDNA clone cES3, a poly (A) tail was found 94 nucleotides downstream of the stop codon. No putative eukaryotic polyadenylation signal (AATAAA) was seen in the 3' non-coding region.

The Esyn protein

The predicted protein product of the *esyn1* gene contains 3131 amino acids and has a molecular mass of 346 900 Da. This agrees with recent estimates of the molecular mass of Esyn of about 350 000 Da (A. Stindl, personal communication).

A computer analysis of possible repeated domains revealed two domains, each of about 420 amino acids. The first domain, A in EA, covered amino acids 499–918 and the second, A in EB, 1572–1988 (Fig. 3C). These domains showed 39.5% similarity and 25% identity when analysed by PALIGN. A protein data base search by FASTA identified these regions as parts of domains of about 600 amino acids which are highly conserved and share homologies with other multifunctional peptide synthetases and adenylate-forming enzymes. The *N*-terminal domain of Esyn covering amino acids 499–1074 (domain EA in Fig. 3C) is similar to domains of gramicidin synthetase A (Krätzschmar *et al.*, 1989) and tyrocidine synthetase A from *B. brevis* (Weckermann *et al.*,1988), ACV synthetase from *Penicillium chrysogenum* (Smith *et al.*, 1990, Diez *et al.*, 1990); *Aspergillus nidulans* (MacCabe *et al.*, 1991) and *Cephaloporium acremonium* (Gutierrez *et al.*, 1991), and enzymes involved in enterobactin synthesis in *Escherichia coli* (Rusnak *et al.*, 1989; 1991). Inspection of the *C*-terminal region of Esyn revealed that the conserved region of EB encoding 420 amino acids is followed by a 434-amino-acid portion (amino acids 1988–2422, named M in Fig. 3C) which showed no similarity to any region of known peptide synthetases. Downstream of this region is a 143-amino-acid portion (2423–2566) which is homologous to the carboxyl parts of these defined conserved domains (labelled X in Fig. 3C). The *C*-terminal 48-amino-acid portion of the domain is repeated, with 54% identity, and a similar region followed EA (labelled S in Fig. 3C). The *C*-terminal 48-amino-acid portions, repeated in Esyn domain EB, encode the conserved LGGXS sequence which is suggested to be involved in covalent binding of

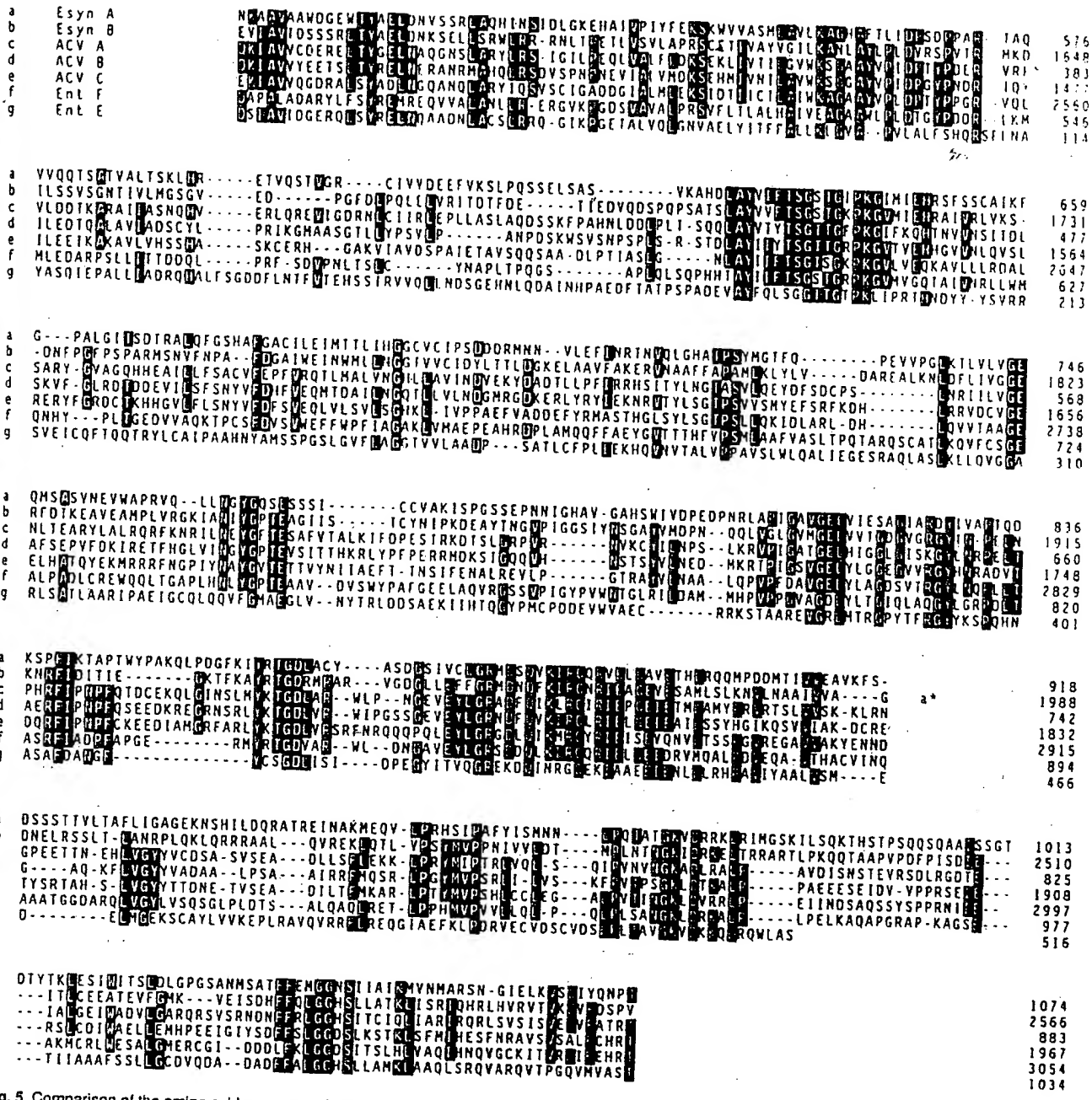

Fig. 5. Comparison of the amino acid sequence of Esyn domains EA and EB with the three domains (A, B, C) of ACV synthetase of *P. chrysogenum* (Smith *et al.*, 1990) and EntF and EntE of *E. coli* (Rusnak *et al.*, 1991; Staab *et al.*, 1989). Conserved amino acids are boxed. a* indicates the 434-amino-acid insertion in Esyn domain EB.

substrate amino acids (Schlumbohm *et al.*, 1991). The positions of all these domains are indicated in Fig. 3C.

With the assistance of CLUSTAL the core regions of similarity between Esyn domains EA and EB were aligned with each of the three domains (A, B, C) of ACVS from *P. chrysogenum* (Smith *et al.*, 1990) and the serine-(EntF) (Rusnak *et al.*, 1991) and 2,3-dihydroxybenzoate-activating (EntE) (Staab *et al.*, 1989) enzymes involved in the biosynthesis of the siderophore enterobactin in *E. coli*, as shown in Fig. 5.

When the amino acid sequence of the internal region M of domain EB was compared, by FASTA, with published protein data banks, no significant sequence similarities were found to known proteins. Only one local similarity was noted in the *N*-terminus that is apparently conserved within adenine- and cytosine-specific DNA and RNA

Table 1. A common region in S-adenosylmethionine-dependent methyltransferases.

Miscellaneous	Sequence	Position	Reference
Esyn	V L E I G T G S G M I L F N L	2086–2100	This work
Ery G	V L D V G F G L G A Q D F F W	85–99	Haydock *et al.* (1991)
Uroporphinogen III MT	V V L V G A G P G D A G L L T	218–232	Blanche *et al.* (1991)
o-Asp/L-isoAsp MT	A L D V G S G S G I L T A C F	81–95	Ingrosso *et al.* (1989)
Protein carboxyl MT	A P D V G S G S G I L T A C F	83–87	Sato *et al.* (1989)
Hydroxyindole MT	I C D L G G G S G A L A K A C	182–196	Ishida *et al.* (1987)
rRNA methyltransferases			
ermE	V L E A G P G E G L L T R E L	65–79	Tuan *et al.* (1990)
ermF	V L D I G A G K G F L T V H L	37–51	Rasmussen *et al.* (1986)
ermA	V V E A G P G E G L L T R E L	62–76	Roberts *et al.* (1985)
Adenine DNA methyltransferases			
PstI	I L D A G A G V G S L T A A F	61–75	Walder *et al.* (1984)
Dam	L V E P F V G A G S V F L N T	31–45	Brooks *et al.* (1983)
*Eco*RV	W V E P F M G T G V V A F N V	35–49	Bougueleret *et al.* (1984)
Cytosine DNA methyltransferases			
DdeI	I I D L F A G C G G F S H G F	3–17	Szmyter *et al.* (1987)
HhaI	F I D L F A G L G G F R L A L	14–28	Caserta *et al.* (1987)
*Eco*RII	F I D L F A G I G G I R K G F	98–112	Som *et al.* (1987)

methyltransferases. The sequence VLE/DXGXGXG has been suggested as a possible component of a binding site for *S*-adenosylmethionine (Ingrosso *et al.*, 1989; Lauster, 1989). The glycine-rich motif is a common element in the sequences of AdoMet-dependent methyltransferases, as shown in Table 1.

The region of about 500 amino acids between Esyn domains EA and EB (1056–1556) is 25% identical to a portion of ACV synthetases from fungi connecting domains B and C. Additional comparisons with regions outside the Esyn domains EA and EB did not reveal any significant homologies.

Discussion

We have described a fungal gene encoding an *N*-methyl peptide synthetase involved in depsipeptide synthesis. The predicted amino acid sequence of the entire *esyn1* gene of *F. scirpi* indicates a novel domain structure and agrees with the results from investigations of the mechanism of enniatin synthesis.

Northern hybridizations revealed one ORF of about 9.5 kb in the genomic clone DASH ES1 without any evidence of a close linkage of other genes near to *esyn1*. No evidence was found for introns in the *esyn1* gene as for several other genes in filamentous fungi, including the *acvA* genes encoding the multifunctional ACV-tripeptide synthetases from *P. chrysogenum* (Smith *et al.*, 1990; Diez *et al.*, 1990), *A. nidulans* (MacCabe *et al.*, 1991) and *C. acremonium* (Gutierrez *et al.*, 1991). These *acvA* genes are closely linked to other genes involved in β-lactam biosynthesis and a bacterial origin of the whole gene cluster has been discussed (Ramon *et al.*, 1987; MacCabe *et al.*, 1990). The presence of introns in the *esyn1* gene seems unlikely because nucleotide sequence analysis did not reveal any regions with a low coding potential and the estimated size of *F. scirpi esyn1* mRNA of about 9500 bases agrees with the length of the ORF, but small in-frame introns containing no termination signals cannot be excluded.

The formation of enniatins and cyclosporins proceeds via a thiotemplate mechanism that additionally includes *N*-methylation steps. The existence of two different sites in enniatin synthetase responsible for binding D-HIV and the amino acid has been deduced from inhibition studies using iodoacetamide and isovaleric acid (Zocher *et al.*, 1982; 1983), and from immunological studies using Esyn-specific monoclonal antibodies (Billich *et al.*, 1987). These findings agree well with the organization of the predicted Esyn protein sequence, which consists of two domains which are less homologous to each other (25% identity) than the three domains of ACVS from *P. chrysogenum* (38–43% identity) (Smith *et al.*, 1990) and the four domains of *B. brevis* GrsB synthetase (45–50% identity) (Turgay *et al.*, 1992). The extent of similarity of *N*-terminal parts of Esyn domains EA and EB corresponds to data obtained from *entF* and *entE* genes of *E. coli* involved in enterobactin biosynthesis (21% identity to each other). Enterobactins are macrocyclic trilactones consisting of three molecules each of 2,3-dihydroxybenzoate and L-serine. EntF activates L-serine and binds it as a thioester.

whereas EntE only catalyses the adenylation of 2,3-dihydroxybenzoate (Rusnak *et al.*, 1989; 1991). It is suggested that in the case of enterobactin the rather low homology between the two enzymes is caused by the fact that EntE is responsible for activation of an aromatic carboxylic acid. In the case of enniatin one domain of Esyn is involved in amino acid activation while the second activates D-HIV.

Previous experiments on limited proteolytic digestion of enniatin synthetase from *F. scirpi* resulted in a 200 kDa fragment which could be detected by two different monoclonal antibodies, mAb 21.1 and mAb 25.91, directed against Esyn (Billich *et al.*, 1987). This protein fragment catalysed the activation and thioester formation of D-HIV and could not activate and methylate the amino acid residue. The *N*-terminus was found to be blocked against Edman degradation, like the *N*-terminus of Esyn itself (R. Pieper and R. Zocher, in preparation). Comparing these data with the results of immunological investigations of fusion proteins encoded by the *esyn1* gene and the predicted amino acid sequence, the *N*-terminal part of Esyn encoding domain EA could be involved in D-HIV activation and thioesterification while the *C*-terminal part of Esyn would be necessary for activation of the amino acid. From data on the organization of gramicidin synthetase B it was proposed that the homologous domains each responsible for catalysing the activation of one substrate amino acid are colinearly arranged in the order of amino acid incorporation into gramicidin S (Turgay *et al.*, 1992).

The Esyn regions responsible for recognizing mAbs 25.91 and 21.1 are separated by at least 615 amino acids spanning a 500-amino-acid portion of domain EA. Both mAbs were shown to inhibit L-valine thioester formation to a different extent (Billich *et al.*, 1987). These data indicate that the two amino acid portions recognized by different mAbs lie close to each other on the native enzyme, resulting in an interference of the enzyme-bound mAbs with the ability of Esyn to thioesterify L-valine.

The main differences between microbial peptide synthetases and an *N*-methyl peptide synthetase were observed in the organization of the *C*-terminal domain of the Esyn protein which differs structurally from known peptide synthetase domains. A 434-amino-acid portion which showed local similarity to *S*-adenosylmethionine-dependent methyltransferases is inserted into domain EB. The deduced molecular mass of about 46 000 Da of this region is in the range of the M_r values of methyltransferases of diverse origin (25–59 000; Lauster, 1989; Ingrosso *et al.*, 1989). From photoaffinity labelling of enniatin synthetase using AdoMet followed by limited proteolysis and methyltransferase inhibitor studies, a single methyltransferase domain was proposed (Billich and Zocher, 1987).

Identification of valine- and leucine-binding peptides of GrsB revealed that the conserved core sequence LGGXS within the carboxyl terminus of the conserved domains might be the site of covalent binding of substrate amino acids (Schlumbohm *et al.*, 1991). This motif is conserved in every domain of peptide synthetases except EntE and resembles binding sites of a 4'-phosphopantetheine cofactor in acyl carrier proteins of fatty acid synthases (Wiesner *et al.*, 1988) and polyketide synthases (Sherman *et al.*, 1989; Cortes *et al.*, 1990; Beck *et al.*, 1990). In non-ribosomal peptide synthesis it was suggested that the 4'-phosphopantetheine cofactor is involved in thioester formation with amino acid substrates and in peptidyl transfer (Gevers *et al.*, 1969). Until now no detailed information was available concerning the exact number and role of 4'-phosphopantetheine cofactors in non-ribosomal peptide synthesis. Besides the LGGXS motifs in ACVS from *A. nidulans* one putative 4'-phosphopantetheine-binding site was proposed (MacCabe *et al.*, 1991) whereas in GrsB from *B. brevis* two sites were detected by sequence comparisons with the 4'-phosphopantetheine-binding motif of acyl carrier proteins (Turgay *et al.*, 1992). Inspecting the Esyn protein sequence for putative 4'-phosphopantetheine-binding sites besides the LGGXS motifs, two sites near the *N*-terminus were identified (amino acid positions 104–115 and 162–173) which showed only a little homology to conserved motifs. Enniatin is synthesized via D-HIV-MeVal dipeptidol intermediates which are thioesterified (Zocher *et al.*, 1983). At the *C*-terminal end of Esyn domain EB the LGGXS motif is repeated, accompanied by a cysteine. These motifs might be involved in binding and assembly of dipeptidol units.

The availability of the *esyn1* gene sequence of *F. scirpi* should facilitate further work to determine the molecular dissection of a peptide synthetase which catalyses the formation of methylated peptides.

Experimental procedures

Microorganisms and culture conditions

F. scirpi Lambotte et Fautrey ETH 1536/J5 was grown in submerged culture using a complete medium (Zocher and Kleinkauf, 1978). *E. coli* strains JM109 (Yanisch-Perron *et al.*, 1985) and XL1-Blue (Bullock *et al.*, 1987) were used as hosts for plasmids derived from pUC8 (Messing and Vieira, 1982) and pBluescript (Stratagene), respectively. *E. coli* strain pop 2136 was used as a host for plasmids derived from pEX1, pEX2 and pEX3 (Stanley and Luzio, 1984). *E. coli* strain P2392 (Frischauf *et al.*, 1983) was used for the propagation of phage Lambda DASH (Stratagene). *E. coli* strains Y1088 and Y1090 were used as hosts for phages derived from λgt11 (Young and Davis, 1983).

Constructing and screening a cDNA library

First- and second-strand cDNAs were synthesized from poly

(A)$^+$ RNA of *F. scirpi* using a cDNA synthesis kit and oligo dT(12-18) as a primer (Amersham). cDNAs extended with *Eco*RI adaptors were ligated to λgt11 arms and the phages were packaged *in vitro*, resulting in 1.5×10^5 recombinant plaques. The cDNA was amplified in *E. coli* Y1088 and screened by the method of Young and Davies (1983) with anti-Esyn antiserum, raised in rabbits. The monospecific serum was purified by exposing it to lysates of *E. coli* Y1090 immobilized by reaction with cyanogen-bromide-activated Sepharose 4B (Pharmacia). Phages expressing immunoreactive proteins were detected by treatment with second anti-rabbit IgG-AP conjugate (Promega). Nitro Blue Tetrazolium (NBT) and 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) were used as substrates (Sambrook *et al.*, 1989). Positive plaques were further purified for three more rounds. The cDNA inserts cES2 and cES3 were subcloned in the *Eco*RI sites of pBluescript SK+.

DNA cloning

Standard DNA techniques for cloning and Southern hybridizations were as detailed in Sambrook *et al.* (1989). For the construction of a genomic *F. scirpi* library in Lambda DASH (Stratagene) as a vector, *F. scirpi* DNA was partially digested with *Mbo*I. DNA fragments with average lengths of 13–20 kb were ligated into the *Bam*HI site of Lambda DASH. The genomic library was screened with a radiolabelled *Eco*RI fragment containing the cES3 clone. A clone extending about 11 kb from the 3' end of the *esyn1* gene DASH ES1 (insert size 16 kb) was used for further subcloning and sequencing. Plasmid pC6.7S was obtained by cloning the 7.1 kb *Sal*I fragment of DASH ES1 in the correct orientation into the *Sal*I site of pUC8. Subcloning the 1.3 kb *Hin*dIII (blunted)–*Sal*I fragment in the *Sma*I–*Sal*I sites of pBluescript SK+ and further transfer of the *Bam*HI–*Sal*I fragment into the corresponding restriction sites of pEX3 resulted in pX1.4H. Cloning of the 1.95 kb *Sal*I–*Pst*I fragment into the corresponding sites of pEX2 resulted in pX2.1S; a 1.88 kb *Bam*HI fragment derived by partial *Bam*HI digestion of pC6.7S was cloned in pEX2 (pX1.9B); cloning of a 3.3 kb *Bam*HI–*Sal*I fragment in pEX3 resulted in pX3.2B. The cES3 fragment was subcloned into the *Eco*RI site of pEX2. The subcloned DNA fragments used in expression studies are indicated in Fig. 3B.

Expression of fusion proteins and Western blotting

Heat-induced expression of proteins from *E. coli* strain pop 2136 harbouring pEX1,2,3 derivatives was achieved at 40°C for 2.5 h. *E. coli* strain JM109 transformed with pC6.7S was induced for 3 h with 1 mM IPTG at 37°C. Crude extracts were performed by the freeze and thaw method (Sambrook *et al.*, 1989) in the presence of 1% SDS to achieve soluble overexpressed proteins. Protein extracts were separated by SDS–PAGE in 5% (w/v) Laemmli gels. Electrophoretic transfer of the polypeptides to nitrocellulose and immunoblotting were performed as described by Billich *et al.* (1987). Anti-rabbit IgG-AP and anti-mouse IgG-AP conjugates (Promega) were used as second antibodies. Substrates were used as described above.

DNA and RNA isolations

DNA was isolated as described by Specht *et al.* (1982) from mycelium of *F. scirpi* grown for 72 h in complete medium. Total RNA of a 72 h culture of *F. scirpi* was isolated using the procedure of Flurkey and Kolattukudy (1981). Poly (A)$^+$ RNA was then isolated from total RNA by chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972). Electrophoresis of RNA and Northern blotting were carried out as described by Ausubel *et al.* (1987).

Sequence analysis

Fragments of the *esyn1* gene from the Lambda clone DASH ES1 and plasmid cES3 were subcloned in pBluescript SK+ and KS+ and sequenced using KS, M13 and reverse oligonucleotides (Stratagene) as primers. Plasmid DNA was purified with Quiagen columns and sequenced directly (Chen and Seeburg, 1987) with the T7 sequencing kit from Pharmacia using the chain termination procedure (Sanger *et al.*, 1977). The complete sequence of both coding and non-coding strands were obtained. Computer programs supplied as part of the PC Gene sequence analysis package (University of Geneva, Switzerland) were used for sequence analysis and comparisons. The SWISSPROT and NBRF protein databases were searched using the program FASTA of Pearson and Lipman (1988). The nucleotide sequence data reported in this paper will appear in the EMBL nucleotide sequence databases under the accession number Z18755.

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